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RECOGNITION AND PHAGOCYTOSIS OF APOPTOTIC CELLS

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Abstract

Physiological elimination of unwanted cells within the organism occurs via cell death by apoptosis and phagocytosis of these cells represents a key event in the apoptotic process. Macrophages, which are the dedicated phagocytes, and other occasionally phagocytic cells ingest the apoptotic cells while they are still intact, thus preventing the leakage of potentially harmful materials from the dying cells. Although evidence has been presented that the elimination of apoptotic bodies from the tissue operates by means of specific recognition systems, the molecular mechanisms by which an apoptotic cell is recognized are poorly understood. Recent data indicate that phagocyte recognition of apoptotic cells involves at least four classes of receptors on the phagocyte surface. On the other side, dying cells may display different signals to signal their status. Exposure of phosphatidyl serine (PS) on the surface of apoptotic lymphocytes triggers their specific recognition and removal by macrophages. Apoptotic thymocytes are also identified by altered lipid packing on their surface. Different populations of macrophages use either the vitronectin receptor or the PS receptor to recognize and remove apoptotic cells. It has been suggested that the asialoglycoprotein and the galactose-specific receptors of healthy hepatocytes and sinusoidal liver cells are implicated in the engulfment of apoptotic hepatocytes, likely in cooperation with other hepatic carbohydrate-specific receptor systems. The purpose of this review is to examine current knowledge of the mechanisms by which phagocytes recognize and ingest apoptotic cells.

Key Words: Apoptosis, hepatocytes, Kupffer cells, endothelial cells, phagocytosis, galactose-specific-receptors, mannose-specific receptors.

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Introduction

One of the characteristic features of apoptosis *in vivo* is that cells undergoing this programmed form of death are rapidly recognized and removed by phagocytic cells. Apoptosis is a physiological and "programmed" form of cell death, that was described, more than 20 years ago, morphologically as a phenomenon distinct from the "accidental" cell death or necrosis (Kerr *et al.*, 1972). This process plays an essential role in normal tissue homeostasis as well as in certain pathological conditions, since it is responsible for deletion of unwanted cells (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Duvall and Wyllie, 1986; Cohen, 1991). Apoptosis, as a programmed cell death process, can be described by a distinct set of morphological and biochemical features (Martin *et al.*, 1994) and can be induced in many cell types via numerous physiological, biochemical and/or noxious stimuli (Schwartzman and Cidlowski, 1993).

The biochemical and morphological characteristics of apoptosis have been extensively described and the morphological appearance of apoptotic cells such as cell shrinkage, chromatin condensation or fragmentation into membrane-bound apoptotic bodies is well known (Fig. 1). These modifications are accompanied by biochemical changes such as internucleosomal cleavage of chromatin into low molecular weight fragments. Much of the recent interest undoubtedly stems from biochemical studies of apoptosis: those concerned with understanding its initiation and regulation and those aimed at evaluating the processes that underlie the stereotyped sequence of morphological events observed by electron microscopy. A great deal of effort has been dedicated to studies in the above fields, while less interest has been shown in the study of the mechanisms undergoing apoptosis *in vivo*; in particular, few studies have focused their attention to dissect the physiological fate of the dying apoptotic cells. The phagocytosis of apoptotic cells and/or bodies by local phagocytes leads to the ingestion of the intact apoptotic cells before their lysis, thus preventing damage to neighboring cells or eliciting an inflammatory response (Wyllie *et al.*, 1980; Duvall and Wyllie, 1986;

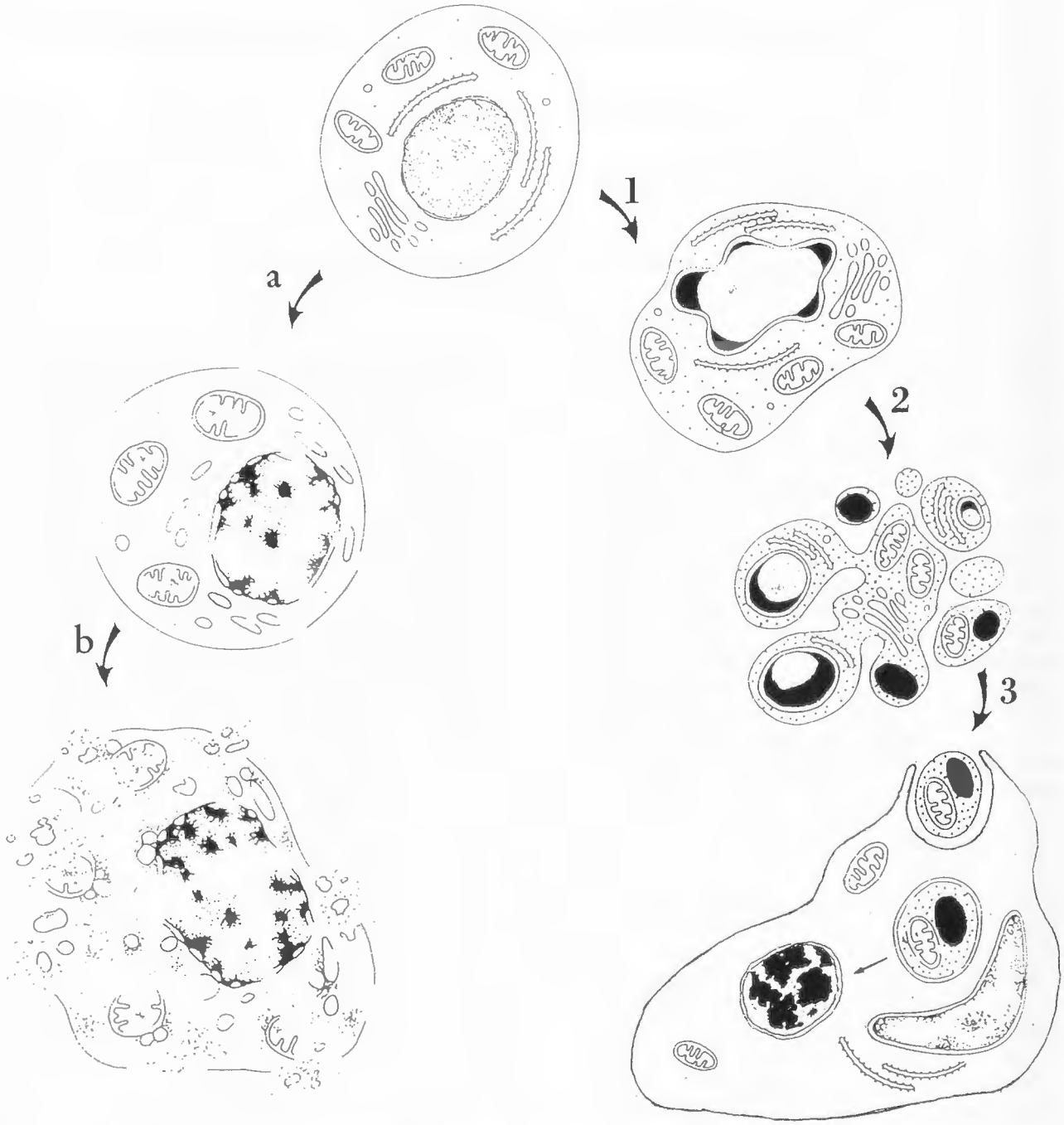

Figure 1. Schematic representation of the sequence of ultrastructural changes that takes place in apoptosis (right) and necrosis (left). A normal cell is represented at the top. The early stages of apoptosis (1) are characterized by chromatin compactation and segregation into sharply delineated masses on the nuclear envelope, condensation of the cytoplasm and mild convolution of the nuclear and cellular outlines. Rapid progression of the latter two processes (2) is then associated with marked convolution of the cell surface, breaking-up of the nucleus into discrete, membrane-enclosed fragments in which the segregation of compacted chromatin is maintained, and budding of the cell as a whole to produce membrane-bounded apoptotic bodies which are recognized and phagocytosed by adjacent cells (3). The onset of necrosis in irreversibly injured cells (a) is characterized by clumping of chromatin, with ill-defined edges, without radical change in its distribution. Simultaneously, all cytoplasmic compartments undergo marked swelling, densities appear in the matrix of mithocondria, and membranes begin to break down. At a later stage (b), membranes break down and nuclei and organelles disintegrate.

Batsford, 1991; Bell and Morrison, 1991). Macrophages, as well as other cell types, can participate to internalize apoptotic cells in a very efficient manner. In fact, in tissue sections, apoptotic cells and/or bodies or their undigested traces can be seen inside large phagosomes in healthy cells. Since the processes of ingestion seem to be very rapid, is likely that many cells are removed by this mechanism without been detectable.

The phagocytosis of apoptotic cells has, therefore, a potential protective role that needs to be investigated further. In fact, this event defends an organism against potentially harmful contents of dying cells. Defects in clearance of apoptotic cells may be a previously unrecognized pathway leading to disease. The final intracellular fate of intact ingested cells undergoing apoptosis is the lysosomal enzyme destruction of potentially toxic substances. This mechanism does not alter the normal physiological status of the surrounding tissue. This peculiarity is well documented during *Drosophila* development, where a large number of cells are eliminated by apoptosis (i.e., approximately one half of the cells in the developing central nervous system, or thousands of unwanted interommatidial cells) and are rapidly phagocytosed by macrophage-like blood cells without disrupting the architecture of the organs. Interestingly, *Drosophila* macrophages share some of the biochemical characteristics of their mammalian counterparts, such as scavenger receptor activity (Abrams *et al.*, 1992).

However, the mechanisms by which macrophages recognize apoptotic cells in *Drosophila* or in other organisms remains to be determined. It should be possible to identify genes involved in this process by screening for mutations that cause defective engulfment of apoptotic cells (White and Steller, 1995). In the nematode *Caenorhabditis (C.) elegans*, mutations in any of seven different genes can result in impairment of ingestion of cells dying by programmed death (Robertson and Thomson, 1982; Hedgecock *et al.*, 1983; Sulston *et al.*, 1983; Ellis *et al.*, 1991a, 1991b).

That the phagocytosis of apoptotic cells is an important and physiologically widely diffuse process is also supported by findings of phagocytosis in tissues where it was never assumed to be necessary. In fact, Han (1993) has recently demonstrated that macrophages are aggregated at the villus tips of the small intestine of different mammals and phagocytose effete apoptotic enterocytes, in contrast to the accepted view that enterocytes are exfoliated into the lumen.

As all the above data indicate, multiple signals are necessary for a phagocyte to determine that an intact cells is apoptotic, and therefore, mature for removal. Which are the possible recognition mechanisms that act as receptors on the phagocytosing cells and which are the signal of "edible status" on the apoptotic cells? The answers seem to be very difficult and complex. In fact, it appears that phagocytes ingesting apoptotic cells may use one or more of the possible recognition mechanisms. On the other side, apoptotic cells display their conditions in a number of different ways. The reason why cells undergoing apoptosis can display different ways to signal their status is still unknown. The possibility of parallel and partially redundant recognition systems suggested by the study of the *C. elegans* is consistent with the recent finding that mammalian phagocytes may express more than one class of recognition mechanism; this might enhance the capacity of phagocytes, in particular those "non-professional" phagocytes, to take up apoptotic cells. In turn, this could enhance both the probability and rate of apoptotic cells to be promptly removed by neighboring cells, thus preventing any leakage of their undesired contents.

Phagocyte cell surface mechanisms for the recognition of apoptotic cells

To date, at least four different molecular mechanisms, involving different classes of receptor molecules, have been suggested to play a role in the phagocytosis of apoptotic cells and/or bodies. The following classes of phagocyte molecules have been implicated.

Vitronectin receptor integrin/thrombospondin
Macrophage uptake of apoptotic neutrophils (but not of the non-apoptotic control cells) was specifically inhibited by amino sugars and amino acids in a charge-dependent manner (Savill *et al.*, 1989a, 1989b). Variation of the pH at which the interaction was performed resulted in variations of the macrophage uptake, thus suggesting a correlation of phagocytosis with the pH and cationic charge of these small molecules (Savill *et al.*, 1993). The lack of inhibition for the macrophage recognition of apoptotic neutrophils by treatment of aging neutrophils with broad-spectrum proteases or inhibitors of protein synthesis suggests that the nature of these putative neutrophil cell surface molecules seems unlikely to be proteic (Savill *et al.*, 1993). The fact that the $\alpha_v\beta_3$ vitronectin receptor integrin of the macrophage has been indicated as a major contributor in the macrophage phagocytosis of apoptotic neutrophils is derived from studies showing the inhibitory effects of peptides and proteins bearing the Arg-Gly-Asp (RGD) tripeptide adhesion signal and the inhibitory effects of monoclonal antibodies directed against subunits of this integrin (Kay, 1975). This represents an unexpected function for a receptor previously thought only to mediate adhesion of cells to matrix (Pytela *et al.*, 1985), but is consistent with known roles of other integrins in cell-cell interactions (Hynes, 1987). In addition, the vitronectin receptor is not only involved in macrophage recognition of peripheral blood lymphocytes (Savill *et al.*, 1990), thymocytes

(Fadok *et al.*, 1993) and eosinophils undergoing apoptosis (Stern *et al.*, 1992), but it also participates in recognition of apoptotic neutrophils by fibroblasts (Hall *et al.*, 1994) and smooth muscle-like glomerular mesangial cells (Savill *et al.*, 1992b).

However, the ligand on the neutrophil surface that mediates the macrophage $\alpha_v\beta_3$ seemed unlikely to be a RGD-bearing protein but rather a macrophage-secreted trombospondin (TSP). TSP is a multifunctional trimeric, RGD-bearing, adhesive glycoprotein implicated in platelet aggregation, tumor metastasis, embryogenesis, vascular smooth muscle proliferation and other events involving cell-cell and cell-matrix interaction (Lawler, 1986; Silverstein *et al.*, 1989). Adhesive properties of TPS are inhibited by amino sugars and cationic amino acids (Silverstein *et al.*, 1986) and by the monoclonal antibodies to TPS and to the macrophage TPS receptor, $\alpha_v\beta_3$ and CD36 (Pytela *et al.*, 1985; Savill *et al.*, 1992a). The most reasonable mechanism of action of the TSP could be that, once secreted by macrophages into their surroundings, it can form a molecular bridge between the apoptotic neutrophils and the macrophage surface, likely in cooperation with both $\alpha_v\beta_3$ and CD36 (Pytela *et al.*, 1985; Savill *et al.*, 1993).

That the cell surface antigens play an important role in the recognition and ingestion of intact apoptotic cells, has been further supported by the data of Ren *et al.* (1995). They reported that the transfection of the macrophage adhesion molecule CD36 into human Bowes melanoma cells specifically conferred greatly an increased capacity (comparable to that exhibited by macrophages) to ingest apoptotic neutrophils, lymphocytes and fibroblasts. Therefore, CD36 gene transfer can confer "dedicated" capacity to ingest apoptotic cells upon "occasional" phagocytes.

Receptors for phosphatidyl serine An additional mechanism by which phagocytes might recognize and internalize apoptotic cells has been suggested by the observation that macrophages are able to bind to red blood ghosts and sickle-shaped red blood cells (Schwartz *et al.*, 1985; McEvoy *et al.*, 1986). These cells have lost the normal asymmetry of membrane phospholipids, by which the outer leaflet of the membrane bilayer predominantly contains the neutral phospholipids sphingomyelin and phosphatidyl choline, while anionic phospholipids, such as PS, are usually restricted to the inner monolayer. The altered red blood cells display increased amounts of surface PS which, in addition to altering cell surface hydrophobicity and charge, may be recognized by putative macrophage receptors for PS.

The phagocytosis of apoptotic cells, by macrophages elicited into the peritoneal cavity, can be inhibited by PS regardless of the species (human or murine) or type (lymphocyte or neutrophil) or the apoptotic cell (Fadok *et al.*, 1992a). That human macrophages are able, under some conditions, to recognize PS on apoptotic cells was suggested by the observation that PS liposomes inhibit phagocytosis by phorbol-ester treated THP-1 cells (Fadok *et al.*, 1992b). The dramatic changes in the cell-surface structure observed during apoptosis (Morris *et al.*, 1984) suggested that asymmetry of membrane phospholipids might occur in this process. It has been shown recently that murine-elicited macrophages stereospecifically recognize PS exposed on the surface of apoptotic cells. The particulate stimulus, β-1, 3-glucan, stimulated bone marrow-derived macrophages to express several characteristics of inflammatory macrophages and induced these cells to recognize PS on apoptotic cells. Induction of PS recognition in bone marrow-derived macrophages was associated with digestibility of the stimulus, because L, but not D amino acid particles or latex, were able to stimulate macrophage recognition of PS (Fadok *et al.*, 1993). It should be further emphasized that both apoptotic murine thymocytes and cells from the CTLL-T-cell line undergoing apoptosis showed increased exposure of PS when compared with non-apoptotic cells (Fadok *et al.*, 1992b). Therefore, in addition to the vitronectin-integrin receptor, macrophages have specific receptors which enable them to recognize PS, exposed on the surface of apoptotic cells as a signal for phagocytosis and removal (Fadok *et al.*, 1992a).

The loss of phospholipid asymmetry is consistent with a loss of normal lipid packing of cell membrane. Schlegel *et al.* (1993) found that apoptotic thymocytes isolated from mice injected with hydrocortisone and stained with merocyanine 540 (MC 540), a fluorescent probe sensitive to lipid packing, showed, at 9-10 hours after injection, a clear increased MC540 staining in a subpopulation of cells. When DNA degradation was assessed by staining fixed cells with propidium iodide (PI), the fraction of cells with increased MC540 staining corresponded to the fraction with reduced PI staining. These results indicate that living apoptotic thymocytes can be identified and separated on the basis of altered lipid packing and increased staining with MC540. In mature mouse B cells undergoing apoptosis, Mower *et al.* (1994) reported a decreased membrane phospholipid packing together with a decreased cell size; this has been described as an early apoptotic stage.

Uncharacterized macrophage surface antigens Flora and Gregory (1994) have been recently described that the *in vitro* recognition of apoptotic cells by human peripheral blood monocyte-derived macrophages is inhibited by monoclonal antibody 61D3, a widely-used marker of monocyte/macrophage lineage cells. Therefore, the antigen defined by the 61D3 mAb is an important mediator of apoptotic cell recognition. The finding that apoptotic cell recognition pathway specified by

61D3 could be distinguished from that involving the macrophage $\alpha_v\beta_3$ vitronectin receptor provides further evidence that the mechanisms underlying rapid clearance of apoptotic cells involve multiple phagocyte receptors.

Lectin-like receptors A role for the lectin-like receptors in the recognition and phagocytosis of apoptotic cells has been suggested since the study of Duvall *et al.* (1985) on rodent thymocytes induced to apoptosis by glucocorticoids. Mouse peritoneal macrophage binding of mouse thymocytes was inhibited by 20 mM N-acetylglucosamine to about 50% of control levels, whereas the binding of freshly isolated non-apoptotic thymocytes was unaffected by the sugar addition. N-acetyl galactosamine (GalNAc) and galactose were found the only other sugar moieties to exert inhibition. The sugars were blocking a putative lectin-like receptor on the macrophage surface since the binding was reduced when it was pre-incubated with macrophages but not with apoptotic thymocytes.

These data, together with the morphological changes seen in glucocorticoid-induced apoptotic thymocytes observed by electron microscopy (Morris *et al.*, 1984), suggested that, as a consequence of apoptosis, cells undergo specific cell surface modifications including changes in the exposition of surface carbohydrates. The reduced cell electrophoresis migration of apoptotic cells, when compared with non-apoptotic counterparts also suggested the loss of surface negative charges. It could be hypothesized that surface sialic acid residues are stripped out (by an unknown mechanism, probably similar to that exerted by neuraminidase treatment) from the cell surface while the process of apoptosis is going on. Thus, the exposure, on the surface of apoptotic cells, of normally masked residues of N-acetyl glucosamine (GlcNAc), N-acetyl galactosamine and galactose permits their recognition by interaction of sugar moieties and putative macrophage lectins. Despite the lack of the expected differences between apoptotic and non-apoptotic thymocytes in the relative binding of labelled lectins, the data strongly suggested that loss of sialic acid and other carbohydrate changes may be a specific feature of apoptotic cells involved in recognition by other occasional phagocytes, as in the case of phagocytosis of apoptotic hepatocytes by healthy ones (Dini *et al.*, 1992) and of apoptotic neutrophils by fibroblasts (Hall *et al.*, 1994).

Hepatic lectin-like receptors Taking into account the idea that the apoptotic cell surface might expose normally masked sugar residues, rendering them available for interaction with lectin-like receptors on phagocytes, we verified if this was the case for the hepatic cells, in view of the fact that liver cells (hepatocytes, Kupffer and endothelial cells) express an enormous amount of receptors belonging to the sugar recognition systems (i.e., asialoglycoprotein receptor, ASGP-R; galactose-specific receptor; mannose/fucose-specific receptor). Our first demonstration that the ASGP-R (likely in cooperation with other carbohydrate receptors) is involved in the phagocytosis of apoptotic hepatocytes by healthy ones was performed on newborn hepatocyte cultures induced to undergo apoptosis by hormonal treatments (Dini *et al.*, 1992). The apoptotic bodies, floating in the culture supernatant, are removed by the hepatocytes. We observed several immature glycans (N-acetyl galactosamine, mannose and galactose) specifically expressed on the surface of apoptotic hepatocytes derived both from the supernatant of these cultures as well as isolated from livers of rat treated with a single injection of lead nitrate to induce apoptosis (Dini *et al.*, 1993). The ability of the specific receptor antibodies and sugar moieties to block their binding and uptake by the living liver cells, i.e., hepatocytes, Kupffer and endothelial cells, support the conjecture that the galactose-specific receptor is involved in the clearance of apoptotic cells.

In liver, like other organs, apoptosis plays a key role during physiological cellular renewal and in cellular depletion after stimulation with mitogens or hyperplasia-inducing treatments (Dini *et al.*, 1993). In this latter phenomena, liver macrophages, i.e., Kupffer cells, represent the most active cell population in the process of elimination of apoptotic bodies, as shown at electron microscopic level. Due to the modifications of surface glycoconjugates of apoptotic cells, their uptake by Kupffer cells probably occurs through molecular interactions between their carbohydrate-specific receptors and the apoptotic body shell (Dini *et al.*, 1993). It is worth noting that liver endothelial cells also expose carbohydrate-specific receptors on their surface, whose function is linked to receptor-mediated endocytosis of circulating modified glycoproteins, and that the cells show the capability to engulf large-sized materials (Steffan *et al.*, 1986). In addition, on the cell surface of non-apoptotic liver cells, the expression of the ASGP-R, the galactose-specific receptor and the mannose-specific receptor is modulated (enhanced or decreased) during the entire process of apoptosis. Therefore, both hepatocytes and non-parenchymal liver cells could play a role in the recognition and subsequent phagocytosis of cells which undergo apoptosis, and this interaction could likely be dependent upon specific carbohydrate-receptor recognition.

Experimental Procedures

To verify whether the carbohydrate-specific receptors of non-parenchymal liver cells are involved in the phagocytic process of apoptotic hepatocytes and circulating apoptotic cells we used three different experimental models:

Table 1. Number of LacBSA-Au_5 and Inv-Au_5 particles per micrometer of plasma membrane profile on hepatocytes and Kupffer cells after lead-nitrate treatment and percentage of phagocytic activity of Kupffer cells.

days after treatment	hepatocytes		Kupffer cells		percent phagocytosis
	LacBSA-Au_5	Inv-Au_5	LacBSA-Au_5	Inv-Au_5	
control	2.5 (0.1)	1.9 (0.2)	15.9 (2.1)	1.1 (0.4)	0-2
1	2.8 (0.2)	1.2 (0.1)	*9.7 (1.8)	2.8 (0.7)	20
3	*1.2 (0.01)	0.8 (0.05)	*28.8 (3.0)	1.3 (0.3)	43
5	*5.8 (1.2)	1.1 (0.09)	12.1 (1.5)	1.5 (0.5)	31
7	nd	nd	nd	nd	10
10	nd	nd	nd	nd	22
15	*15.3 (1.5)	*2.7 (0.1)	12.9 (1.6)	2.5 (0.6)	34
40	3.5 (0.3)	1.5 (0.09)	12.7 (1.6)	1.1 (0.2)	15
60	2.5 (0.2)	nd	17.7 (1.9)	nd	20

The values are the mean of three different experiments. The standard deviations (s.d.) are given in parenthesis.
*$p < 0.01$ with respect to control value. nd: not determined.
Quantification of phagocytosis was calculated in the electron microscope by counting the number of Kupffer cells showing phagosomes containing condensed material on at least 100 cells for each time point in three different experiments.

In vivo lead nitrate treatment

Lead nitrate was injected intravenously into male Wistar rats in a dose of 10 μmol/100 g of body weight (Columbano *et al.*, 1985). After 1, 2, 3, 5, 7, 9, 11, 13, 15, 30, 40 and 60 days from the injection, the animals, fed with normal lab chow, were sacrificed for the different experiments. Galactose and mannose specific receptors expression were measured by using lactosylated bovine serum albumin (LacBSA; Soeckebord, Uppsala, Sweden) and Invertase (Inv)-gold particles (Au_5; Sigma, St. Louis, MO) in *in situ* and *in vitro* (isolated Kupffer cells and hepatocytes) binding experiments as reported elsewhere (Dini *et al.*, 1993).

Morphometric evaluation of the number of binding sites per micrometer of plasma membrane of hepatocytes and Kupffer cells was done by using a Quantimet 970-Image analysis system (Cambridge Instrum., Cambridge, U.K.). The number of coated pits per endothelial liver cell and number of gold particles per coated pit were also determined at different times after lead nitrate injection by scoring at least 100 cells on the electron microscope.

Phagocytosis between cultured isolated endothelial cells and isolated liver apoptotic bodies

Mouse liver sinusoidal cells were isolated by means of D-collagenase (Boehringer, Mannheim, Germany), 0.05% in Dulbecco's Minimal Essential Medium (D-MEM, Flow, Irvine, Scotland) and pronase (Sigma, St. Louis, MO), 0.1% in D-MEM, perfusion of livers and separated from parenchymal and blood cells through centrifugation in a 30% metrizamide gradient by a modification of the method of Barberà-Guillem *et al.* (1991). The cells (1.3 x 10^6 cells/ml), plated onto 20 cm^2 Petri dishes (4 x 10^6 cells each), were incubated at 37°C for 15, 20, 30 and 60 minutes, with apoptotic bodies (5 x 10^5/ml) isolated from rat livers of animals injected with lead nitrate as described elsewhere (Dini *et al.*, 1993). Inhibition experiments were performed incubating the cells with a solution of galactose, N-acetyl-galactosamine and mannose (Gal/GlcNAc/Man) at different concentrations (1, 2, 3, 5, 10 and 40 mM) for 15, 20 and 60 minutes at 37°C before the incubation with apoptotic bodies.

Phagocytosis between cultured isolated Kupffer cells and human apoptotic lymphocytes

Peripheral blood lymphocytes (PBL) from healthy donors were isolated by differential centrifugation using Ficoll-Paque (Pharmacia, Uppsala, Sweden), resuspended and cultured at a concentration of 2 x 10^6 cells/ml in RPMI 1640 (Flow) medium. Apoptotic cells were obtained by keeping lymphocytes in water baths equilibrated to 43°C for 1 hour followed by 1 hour of recovery. Localization of sugars on the surface of apoptotic lymphocytes was performed by using fluorescent lectin conjugates with different specificities: Concanavalin-A (a-D-mannosyl); *Phaseolus limensis* (N-Acetyl-D-Galactosamine); *Ricinus communis* (D-Galactosyl); *Ulex europaeus* (α-L-fucosyl) (all lectins from Sigma). Rat Kupffer cells, enzymatically isolated by the collagenase

Table 2. Number of coated pits and number of LacBSA-Au_5 and Inv-Au_5 particles per coated pit in endothelial cells after lead-nitrate treatment.

days after treatment	number of coated pit per endothelial cell	number of LacBSA-Au_5 per coated pit	number of Inv-Au_5 per coated pit
control	8.4 (0.9)	8.3 (1.0)	5.7 (0.3)
1	8.4 (0.9)	*5.1 (0.5)	*0.9 (0.1)
3	†6.0 (0.4)	†6.4 (0.3)	*1.3 (0.1)
5	*5.6 (0.3)	†6.6 (0.3)	*2.7 (0.2)
15	*5.0 (0.4)	*4.2 (0.2)	†7.1 (0.8)

The values are the mean of three different experiments. The standard deviations are given in parenthesis.
*$p < 0.01$; †$p < 0.1$

perfusion method as previously described (Dini *et al.*, 1993) were plated in four-well plates at a density of 1 x 10^6 cells per well in RPMI 1640 medium and incubated with the apoptotic lymphocytes for different interval times. Inhibition experiments were performed by pre-incubating the cells with a mixture of glucose, methyl-α-mannopyranoside, N-acetyl-galactosamine and fucose for 10 minutes at final concentration of 80 mM, and then adding the same mixture every 15 minutes of the incubation time.

Experimental Data

Following the lead nitrate injection, both liver parenchyma and sinusoids were dramatically modified as assayed by scanning and transmission electron microscopy. Soon after the first days following the injection, all liver cell types responded to the presence of lead nitrate, but to different extent. Hepatocytes became more roundish, and many apoptotic cells were visible both inside the hepatocyte parenchyma and into the sinusoidal lumen. Many of the apoptotic cells probably were derived from Kupffer cells, since the number of Kupffer cells was reduced and the nuclei showed chromatin condensation. The remaining healthy Kupffer cells, often present beneath the endothelial wall and within the space of Disse or between the disconnected hepatocytes, became very active in the phagocytosis of apoptotic cells. Two peaks in the phagocytic activity of Kupffer cells were observed: the first between the third and the fifth day from the injection, the second around the fifteenth day (Table 1). Interestingly, endothelial cells also participated in the removal of apoptotic cells, as shown by the presence of many cytoplasmic phagocytotic vacuoles with undigested materials. Despite rare signs of apoptosis and a reduced number of fenestrae that were sometimes observed, the endothelial lining cells appeared almost healthy. During the days following the treatment, further progressive modifications of the liver were found such as the presence of fibrotic materials, many destroyed sinusoids and enlarged spaces between hepatocytes. Small necrotic parenchymal areas were also observed, but only at the longest time from the injection.

During the onset of apoptosis induced by a single intravenous injection of lead nitrate, both the galactose and the mannose specific receptor expression were modulated as reported in Tables 1 and 2. The modification is related to the number of binding sites expressed on the cell surfaces for both carbohydrate recognition systems. However, the intensity and the persistence of the modulation are specific for the different liver cell types, thus indicating different (time and modality) involvement during the process of apoptosis for hepatocytes, Kupffer cells and endothelial cells. The major modifications are related to the asialoglycoprotein receptors of hepatocytes and galactose-specific receptors of Kupffer cells. The former cell type showed a significant decrease of the binding sites at the third day after the lead nitrate injection (which corresponds to the higher increase in total hepatic DNA content according to Columbano *et al.*, 1985) and a progressive increase at the fifth day (which corresponds to the higher number of apoptotic bodies observed in the liver; Columbano *et al.*, 1985) and fifteenth day after the treatment. Kupffer cells showed a decreased number of Lac BSA-Au_5 particles at day 1 after the injection and a doubled number of binding sites at day 3, which corresponded to the higher level of phagocytosis detected for Kupffer cells. The binding activity of mannose-specific receptors showed minor modifications mainly related to day 15 after the injection for the hepatocytes and days 1 and 15 after the injection for Kupffer cells. With regard to endothelial cells, we observed an overall decreasing of binding sites for both galactose and mannose receptors. In normal conditions on endothelial cell surfaces, several coated pits were present, which are the specialized sites where the galactose and mannose binding sites are restricted. We measured a gradual decrease of coated pits on the cell surfaces and a parallel decrease of gold particles on the coated pits, with the only exception for the number of Inv-Au_5 particles at day 15 after lead nitrate injection. In addition, starting from the third day from the beginning of the treatment, we observed a redistribution of the galactose-specific binding sites. A consistent number of gold granules was also found on non-coated areas.

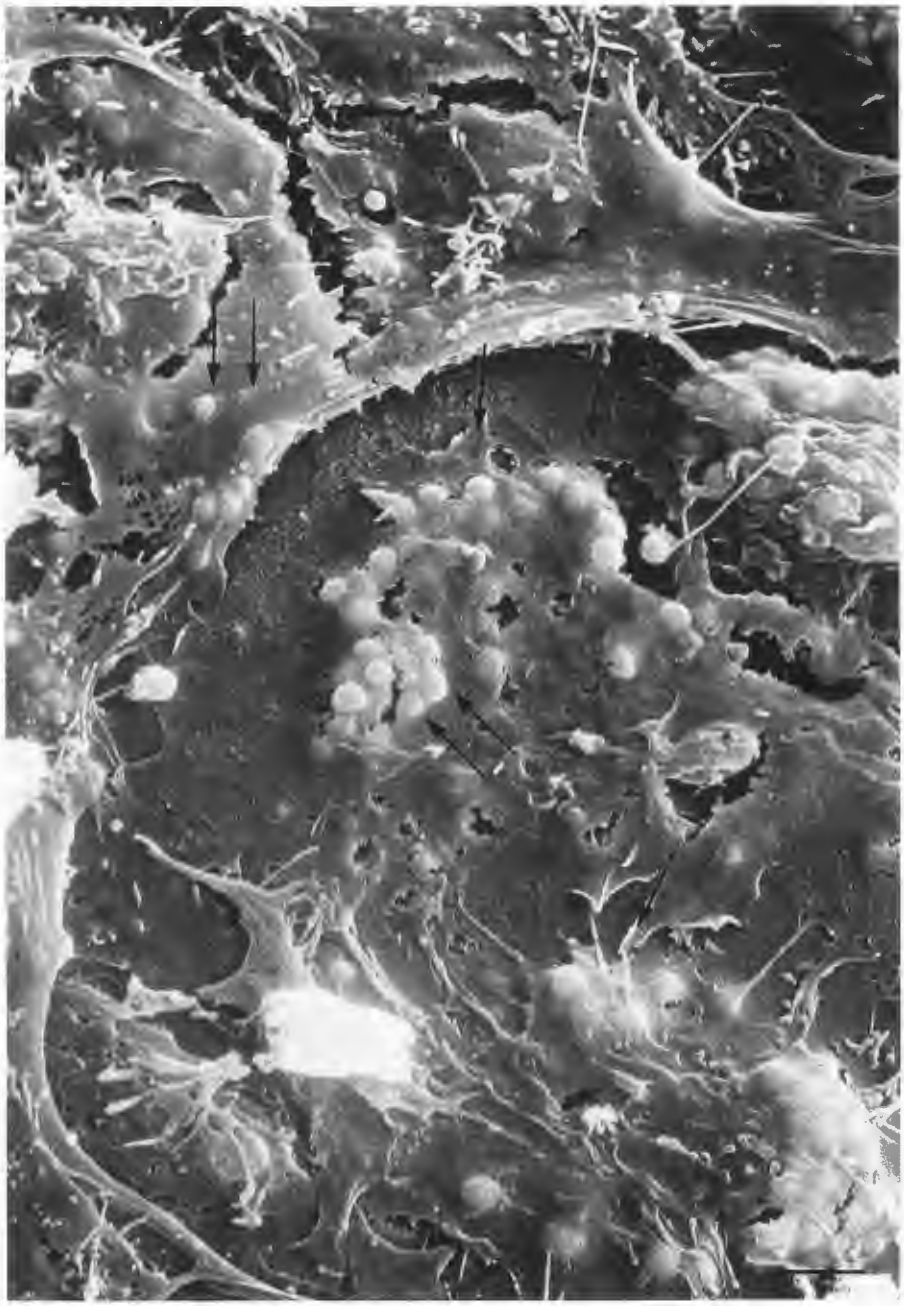

Figure 2. Scanning electron micrograph showing isolated liver endothelial cell phagocyting isolated hepatic apoptotic bodies (arrows). After 30 minutes of incubation, almost all apoptotic bodies were internalized by endothelial cells. Bar = 1 μm.

The meaning of the all changes described above has to be better understood. Accordingly, we are currently studying the modification of hepatic membrane composition in relation to the proliferative and apoptotic stages.

The ability to bind and internalize apoptotic cells and/or bodies was maintained when cultured endothelial cells were incubated with apoptotic bodies isolated from liver (Fig. 2). The binding capacity was assayed in presence and in absence of mixtures of competing saccharides. Ninety percent of the maximum adhesion was obtained within 15 minutes of incubation, reaching the higher percentage of apoptotic body adherence at 30 minutes of incubation. The process of adhesion slowly decreased with time, indicating that apoptotic bodies were removed from the surface due to internalization process which preceded their subsequent degradation (Table 3). A significant reduction (about 50%) of the adhesion percentage, that leads to a reduction in the phagocytic activity (Table 3), was obtained using the mixture of galactose/N-acetyl-galactosamine/Mannose 10 mM. When endothelial cells are treated with interleukins (IL), which increase the sugar receptor expression, an enhanced percentage of adhering apoptotic bodies was measured (Dini *et al.*, 1995).

Table 3. Percentage of isolated rat liver endothelial cells phagocytotic hepatic apoptotic bodies.

treatments		times in minutes 15	20	60
None		5	67	90
(Gal/GlcNAc/Man)	1 mM	6	58	89
(Gal/GlcNAc/Man)	2 mM	4	52	80
(Gal/GlcNAc/Man)	3 mM	5	41	69
(Gal/GlcNAc/Man)	5 mM	2	31	43
(Gal/GlcNAc/Man)	10 mM	2	20	35
(Gal/GlcNAc/Man)	40 mM	1	21*	34
(Saccharose/Ribose)	20 mM	4	62	92

The values represent one typical experiment out of three different experiments performed (*one experiment). Standard deviations do not exceed 10%. The values are given as number of endothelial cells showing phagosomes/number of endothelial cells counted x100. At least 100 cells were counted for each experiment.

Table 4. Fluorescence intensity in normal and apoptotic human lymphocytes and in liver isolated apoptotic bodies, incubated with lectins-FITC.

	CON-A (Man)	PHA (GalNAc)	RCA (Gal)	UEA (Fuc)
Normal lymph.	-	-	+	+
Apoptotic lymph.	+++	+++	++	+
Normal liver cells	-	-	-	+
Apoptotic bodies	+++	+++	+	+++

CON-A: Concanavalin A; PHA: *Phaseolus limensis*; RCA: *Ricinus communis*; UEA: *Ulex europaeus*; Man: mannose; GalNAc: N-acetyl galactosamine; Gal: galactose; Fuc: fucose.
The lectin sugar specificity is given in parentheses.

Peripheral blood lymphocytes, induced to apoptosis by mild hyperthermia (1 hour at 43°C), show, at the electron microscopic level, typical morphological changes: shrinkage of the cytoplasm and condensation of the chromatin that collapses into crescents along the nuclear envelope and then into one or several dense spheres (Fig. 3a). The carbohydrate moieties exposed on the membrane of the apoptotic lymphocytes were very different from that of non-apoptotic lymphocytes, as detected by using both fluorescent or gold conjugated

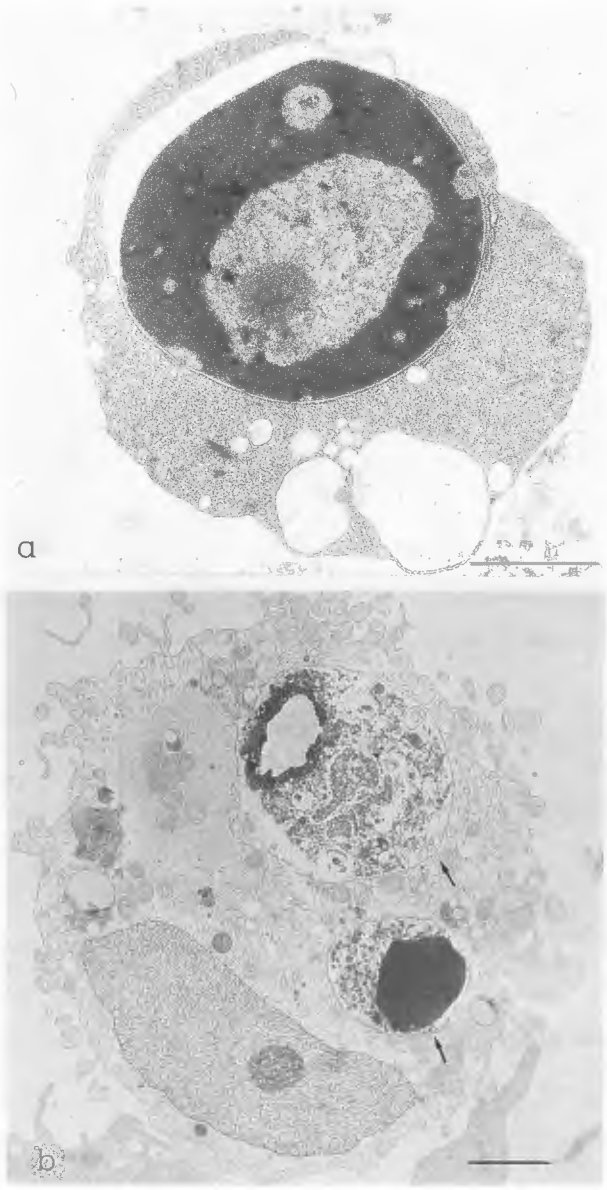

Figure 3. Transmission electron micrographs showing the morphological characteristics of an apoptotic lymphocyte (a) and a Kupffer cell with two phagolysosomes containing apoptotic lymphocyte remnants, with still recognizable nuclear dense masses (b). Bars = 1 μm.

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lectins (Concanavalin-A, *Phaseolus limensis*, *Ricinus communis* and *Ulex europaeus*). In particular, apoptotic lymphocytes, in contrast with normal lymphocytes, specifically expose high levels of mannose, GalNAc and galactose on their surface (Table 4). The apoptotic lymphocytes were rapidly recognized when added to Kupffer cell cultures (Fig. 4). These cells immediately adhered to Kupffer cells and were detected as dark material inside large phagosomes (Fig. 3b). The involvement of

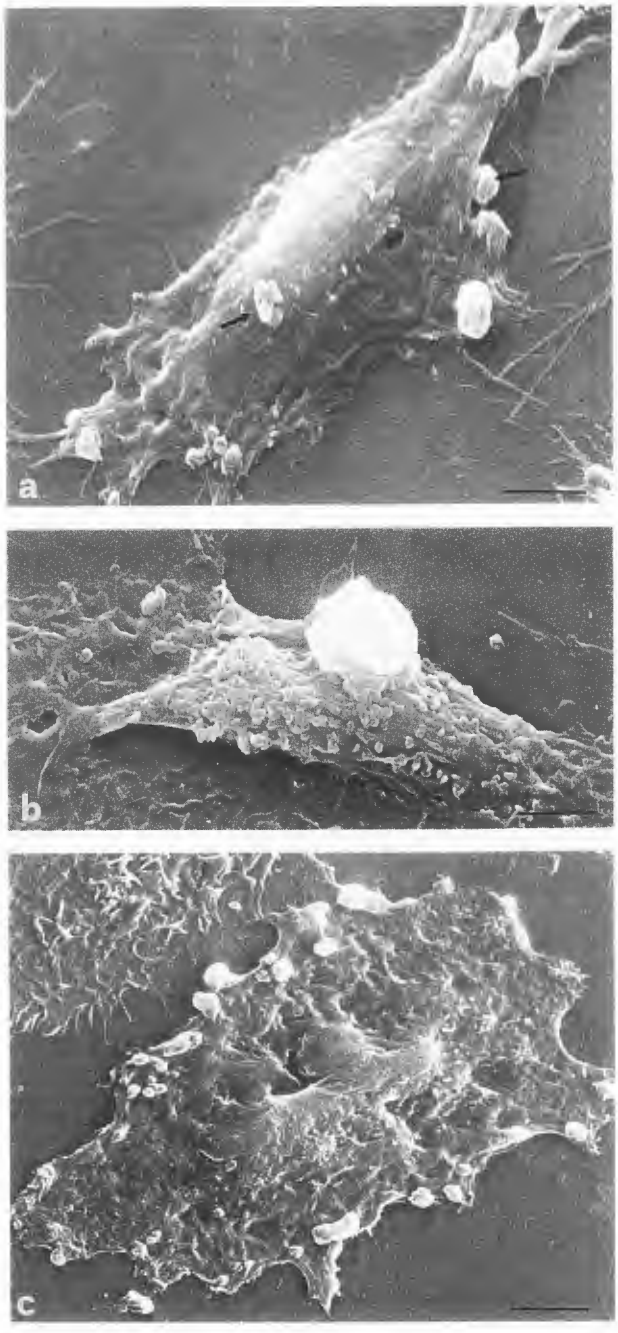

Figure 4. Scanning electron micrographs of cocultures of human apoptotic lymphocytes and Kupffer cells. (a) Apoptotic lymphocytes (arrows) bind to the surface of Kupffer cells; (b) a Kupffer cell phagocyting an apoptotic lymphocyte; (c) after a few minutes, almost all the apoptotic lymphocytes are internalized by Kupffer cells; they are visible as round protrusions of the surface of the macrophages. Bars = 1 μm.

Table 5. Percentage of inhibition of phagocytosis of apoptotic human lymphocytes by isolated Kupffer cells by means of sugar addition.

treatments (80 mM)	times in minutes 5	10	15	30	60
GalNAc	22	42	63	61	62
GlcNAc	27	47	65	56	58
Mixture	75	80	81	80	85
Saccharose/ Ribose*	2	4	3	5	3

The values represent one typical experiment out of three different experiment performed. The standard deviation did not exceed 10%. The sugar mixture contained glucose, N-acetyl-galactosamine, fucose and methyl-α-mannopyranoside at a final concentration of 80 mM. The different inhibitors were added 10 minutes before adding the apoptotic lymphocytes and then every 15 minutes of the culture hour. GalNAc: N-acetyl galactosamine; GlcNAc: N-acetyl glucosamine.

*concentration 40 mM.

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the carbohydrate specific receptors present on Kupffer cells surface was suggested by the fact that there are no visible signs of phagocytosis when Kupffer cells were incubated with a mixture of sugars (glucose, GalNAc, Methyl-α-mannopyranoside and fucose) before addition of the apoptotic lymphocytes. The use of single compounds never reaches the level of inhibition achieved by the sugar cocktail, thus suggesting a cooperation among the galactose and mannose-specific receptors (Table 5). Kupffer cells were never able to bind and internalize non-apoptotic lymphocytes when added to the cultures even at the longer times of incubation.

Conclusion and Future Prospects

The previous brief discussion of the molecular mechanisms involved in the recognition and ingestion of apoptotic cells shows clearly that there is a swift, efficient way of removing unwanted cells from tissue without the release of potentially toxic cell contents, which might otherwise damage neighboring cells and elicit an inflammatory immune response. On the contrary, recently an unfavorable effect on the phagocytosis of apoptotic cells has been reported in the development of AIDS (Kornbluth, 1994). Although apoptosis is often assumed to be a biological dead end, linear, unintegrated retroviral DNA survives apoptosis in avian leukosis virus systems. The viral DNA in apoptotic debris might spon-

taneously transfect macrophages that are avidly phagocytosing apoptosing cells, and thus lead to the production of new virions. Such a hypothetical accessory infection pathway may explain why anti-HIV cytotoxic cells are unable to clear this virus from the body (Kornbluth, 1994).

Despite the increased recognition of the functional relevance of phagocytosis in the process of programmed cell death, little information still exists about the molecular mechanism(s) involved. Until now different mechanisms have been described (lectin-like receptors, $\alpha_v\beta_3$ receptor for vitronectin/integrin/trombospindin,phosphatidyl serine receptor and uncharacterized macrophage surface antigens) to explain in which way a phagocyte can recognize an apoptotic cell, but none is conclusive. Probably there is more than one possible mechanism, and perhaps there can be regional specialization in the recognition process. Maybe a cell undergoing apoptosis displays multiple signal of its status so that the probability of its removal increases, and consequently, the margin of safety for the whole organism is increased. The existence of multiple phagocyte recognition mechanisms for apoptotic cells is not unexpected considering the number of genes involved in removal of cells undergoing programmed death in *C. elegans* (Hedgecock *et al.*, 1983; Ellis *et al.*, 1991a, 1991b) and the fact that, from mutagenic studies, there might be distinct, parallel processes which are partially redundant (Ellis *et al.*, 1991a, 1991b). That these data could be applied to mammalian systems seem unlikely, since, in *C. elegans*, the phagocytosis of dying cells initiates the deletion of cells appearing early in development, generally before any morphological features of programmed cell death are apparent (Robertson and Thomson, 1982). Even if the presence of parallel, partially redundant recognition mechanisms in *C. elegans* would be consistent with the findings suggesting that mammalian phagocytes may express more than one class of recognition mechanism, *C. elegans* does not possess specialized phagocytes resembling those of mammals. For example, fibroblasts recognize apoptotic neutrophils via a vitronectin interaction, but an additional involvement of a lectin-like mechanism was suggested by the inhibitory effects of mannose and fucose (Hall *et al.*, 1994). Cell clearance *in vivo* might depend upon more than one type of phagocyte, each developing a single mechanism as it was described in the inflamed glomerulus where apoptotic neutrophils can be taken up by both macrophages and glomerular mesangial cells (Meagher *et al.*, 1992; Savill *et al.*, 1992b). Therefore, selection of one or more mechanisms for recognition of apoptotic cells by a particular cell type might depend upon the species, the lineage of the apoptotic cell or the nature of the phagocyte involved. It is worth noting that the state of the phagocyte is also particularly important (Savill *et al.*, 1993). Preliminary data indicate that the particular mechanism employed by macrophages may be regulated by external influences (Savill *et al.*, 1993). The exposure of human monocyte-derived macrophages to granulocyte-macrophage colony stimulating factor (GM-CSF), a cytokine known to be present at inflammation sites, increased the recognition of apoptotic human neutrophils (Savill *et al.*, 1993). Cytokines implicated in repair of injured tissue, i.e., transforming growth factor (TGF-β), platelet-derived growth factor (PDGF) and those involved in the initiation of inflammation, i.e., interferon gamma (IFN-γ), interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) also stimulated TPS-dependent recognition of apoptotic neutrophils (Savill *et al.*, 1993). The potential significance of the choice of different recognition mechanism is still a mystery. It is difficult to believe that there are no important reasons why different recognition mechanisms are available to phagocytes.

A further peculiarity is emerging in the complex field of the recognition mechanisms of apoptotic cells. In fact, cooperation in the removal of dead cells is not only restricted to the use of more than one cell surface receptor exposed on the phagocytic cells, but also involves a cooperation among different cellular type sharing the same receptor system for the recognition and removal of apoptotic cells. This fact is well illustrated in the liver where both hepatocytes, Kupffer and endothelial cells operate the plasma clearance of apoptotic cells generated during the involuting phase of liver hyperplasia induced by a single injection of lead nitrate by means of a sugar recognition mechanism (Dini *et al*, 1994, 1995). These data, together with the fact that the phagocytic activity in endothelial cells can be enhanced in macrophage-depleted rats (Bogers *et al.*, 1991) and that IL-1 induces *in vitro* overexpression of mannose-specific receptors on endothelial cells, suggest a cooperation with Kupffer cells in phagocytosis.

Future work will investigate the potential therapeutical use in administering agents to enhance, specifically, phagocytic clearance of apoptotic cells, which could, in turn, reduce tissue injury initiated and/or perpetuated after a persistent inflammation. This idea is consistent with the possibility that deficiencies in clearance of apoptotic cells might be a pathway by which tissue injury is perpetuated, increasing the risk of permanent organ damage, in diseases.

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Discussion with Reviewers

F. Braet: Did you assess liver endothelial cell (LEC) purity?

Authors: We used a modification of the method described by Barberà-Guillem *et al.* (1991) to prepare isolated liver endothelial cells. We used a 30% metrizamide gradient, that is the same percentage extensively used by other groups. The purity of liver endothelial cells was assessed by electron microscopy, while the cell viability was analyzed by using an EPICS-V flow cytometer (Coulter Electronics, Hialeah, FL). In addition, general identification of different sinusoid cell types, in the mentioned paper, was carried out with the following cell markers: ovalbumin (OA), acetylated low density lipoprotein (AcLDL) and rhodamine (Rh-123) for endothelial cells, phagocytosis of latex beads for Kupffer cells.

F. Braet: The endothelial cells obtained in this study are isolated by a pronase/collagenase digestion of the liver. Did pronase treatment affect the number of glycoproteins on the surface of LEC?

Authors: Liver sinusoidal cells were used for all the experiments 48 hours after plating, which allowed for a complete recovery from the procedure of isolation. The same method has also been used in other papers reporting on differences in the lectin binding patterns of subpopulation of liver endothelial cells (Barberà-Guillem *et al.*, 1991; Vidal Vanaclocha *et al.*, 1993).

M. Lanotte: Phagocytosis of apoptotic cells may

influence the "economy" of the organism? It is conceivable that degraded materials generate novel signals when released in tissues?

Authors: Apoptosis is really a major force regulating tissue mass and architecture, and the speed and manner with which cells commit suicide and the rapidity with which their neighbours conceal the evidence, permit no release of inflammatory intracellular components of apoptotic cells into their environment. Since phagocytosis of apoptotic cells is an important aspect of the process of apoptosis, it undoubtedly plays a role in the "social nature" of the programmed cell death within multicellular organisms. The macrophages, beautifully coadiuvated by "amateur" phagocytes work perfectly to eliminate dying cells. If such masses of nutrients (proteins, lipids, sugars, etc.) are re-used by the macrophages or if degradative materials when released into the blood or tissues could generate novel signals, it is not yet elucidated. There is evidence that proliferative and apoptotic machineries of the cell are coupled: every time a cell divides, it activates a suicide programme, that must be suppressed by survival signals if the cell is to survive. Therefore, an obvious extension of this fact is that all cells all the time need signals to prevent a default suicide programme. Whether degradative materials of apoptotic cells belong to this latter class of signals is yet unknown.

M. Lanotte: Knowing the molecules that are used to recognize apoptotic cells for phagocytosis, one can modify unwanted cells to become lure, which then can be engulfed, even when they are not apoptotic. Are macrophages equipped to destroy non-apoptotic cells? Should it be possible to develop techniques based on apoptotic cell recognition to specifically transfer and express genetic material into phagocytic cells?

Authors: Since macrophages are not equipped to destroy non-apoptotic cells, the consequent idea is that the presence of special molecules allows apoptotic cells to be recognized and phagocyted. Up to now, it has been demonstrated that gene transfer of a surface antigen (CD36) can switch "non-professional" phagocytic cells into cells phagocytosing apoptotic cells in a very active manner, like the dedicated phagocytes. The modification of the cell surface of unwanted cells to enhance their removal is still a fascinating possibility that has many implications for the design of new strategies in the therapy of many diseases (e.g., cancer), as the possibility to specifically transfer and express genetic material into phagocytic cells by means of apoptotic cell recognition. Experimental data have shown that the HIV virus can transfect the macrophages that have phagocytosed apoptotic infected lymphocytes.

Reviewer III: I do not think that the "altered lipid packing" described by the Schlegel *et al.* (1993) paper implies any new recognition mechanism.

Authors: The loss of phospholipid asymmetry is consistent with a loss of normal lipid packing of cell membrane. This latter aspect is important, as reported by Schlegel, for the identification and separation of "living" apoptotic lymphocytes. The altered lipid packing could be an early event for the discrimination of living cells and those committed to apoptosis, since it precedes DNA cleavage in mouse B cells.

R.M. Albrecht: The removal of apoptotic cells is **one** physiological process used to eliminate unwanted cells. In the abstract, the authors seem to imply it is **the** (i.e., the only) "physiological" process. Removal of necrotic or otherwise damaged cell by phagocytosis is also physiological.

Authors: The meaning of "physiological process" to eliminate unwanted cells related to the apoptosis results from a decision by the cell based on information from its environment, its own internal metabolism, its developmental history and its genome. The degenerative process of cell death by necrosis, on the other hand, occurs as a result of overwhelming damage of several possible types that allows the cell no such "choice." Unlike cells undergoing necrosis, therefore, cells stimulated to enter apoptosis are often capable of survival but opt to die, presumably for the good of the whole organism and genome; hence, apoptosis could be referred as the "physiological process." Phagocytosis of necrotic or damaged cells is a physiological property of the macrophages; nevertheless, this cause inflammation and further recruitment of other monocyte/macrophages and liberation of cytokines from the phagocytes. On the contrary, when a macrophage phagocytes an apoptotic cell, there is no production of cytokines, and therefore, no further progression of inflammation.

Additional Reference

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